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Hypomethylation of Epstein-Barr Virus DNA in the Nonproducer B-Cell Line EBR

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The conversion of the Epstein-Barr virus-negative Ramos cell line has previously been shown to result in an Epstein-Barr virus-positive non-virus-producer cell line, EBR. We report here that Epstein-Barr virus DNA from EBR alone among several cell lines examined was totally unmethylated at three of four sites containing guanine plus cytosine which were tested. This is in direct contrast to reports of high degrees of methylation in the DNAs of other animal viruses, including herpesviruses, isolated from cells in which the viral genome is expressed at a low level.

Methylation is a postreplicative modification of DNA that results in the modification of about 3% of cytosines in mammalian DNA at the position of the rare dinucleotide 5′-CG-3′ (26). Since DNA methylation in eucaryotes has been found to be tissue and species specific, it has been thought to play a role in the regulation of gene expression (1, 6, 17, 20, 25). The hypothesis that DNA methylation in eucaryotes provides a control mechanism for DNA replication and transcription has been supported in part by the data accumulated on the DNAs of various viruses; actively replicating and transcribing viral DNA has been shown to be hypomethylated, whereas latent inactive viral genomes are highly methylated in comparison, as is the host cell DNA (6). The DNAs of herpesvirus saimiri (5), adenovirus (10, 24), and simian virus 40 (7) and of the proviruses of mouse mammary tumor virus (4) and avian sarcoma virus (9) have been shown to be hypomethylated in producer cell lines and in the virion, but highly methylated when viral DNA is present as nonproductive, latent, or inactive genomes. Recently, Kintner and Sugden (12) have reported that B958 Epstein-Barr virus (EBV) DNA becomes methylated in transformed B lymphocytes after 200 cell divisions following infection.

We report here that EBV DNA is highly methylated at several sites when tested in the nonproducer Raji cell line and that the producer cell lines HR-1 and B958 show a mixture of methylated and nonmethylated viral genomes. However, in contrast to the instances of DNA methylation discussed above, the EBV genomes of the nonproducer EBR cell line showed methylation at only one of the sites containing cytosine-guanine (C-G) that were tested.

In the experiments reported here, the two virus producer B-cell lines used were HR-1 and B958. HR-1 produces non-transforming viral particles (11). B958, which produces transforming viral particles, was originally derived by transformation of marmoset lymphocytes with EBV isolated from a patient with infectious mononucleosis (14). The HR-1 and B958 virus producer cultures used for these experiments were in the range of 3 to 5% positive for EBV viral capsid antigen by indirect immunofluorescence procedures. The nonproducer, EBV-positive B-cell line Raji was derived from an African Burkitt lymphoma biopsy (16). The nonproducer EBR cell line was obtained by the conversion of the EBV-negative Ramos B-cell line to an EBV-positive cell line via HR-1 viral infection (8).

Whole cell DNA was isolated from these cell lines, purified, and subjected to methylation-specific restriction enzyme digestion (see the legend to Fig. 1). The digested DNAs were electrophoresed, blotted onto nitrocellulose filters, and hybridized with ^{32}P-labeled HR-1 EB virion DNA as probe to compare the methylation of EBV DNA in these cell lines at various C-G-containing sites. In the autoradiogram (Fig. 1), *Msp*I and *Hpa*II were used to digest Ramos, EBR, Raji, HR-1, and B958 whole cell DNA. *Msp*I cuts at CCGG sites whether or not the internal cytosine is methylated, whereas *Hpa*II cuts at CCGG sites only if the cytosine adjacent to the guanine is unmethylated on both strands. A comparison of the degree of digestion by this pair of enzymes is a good measure of the degree of methylation of this C-G site within the DNA (3, 13, 22, 27). DNA fragments resulting from

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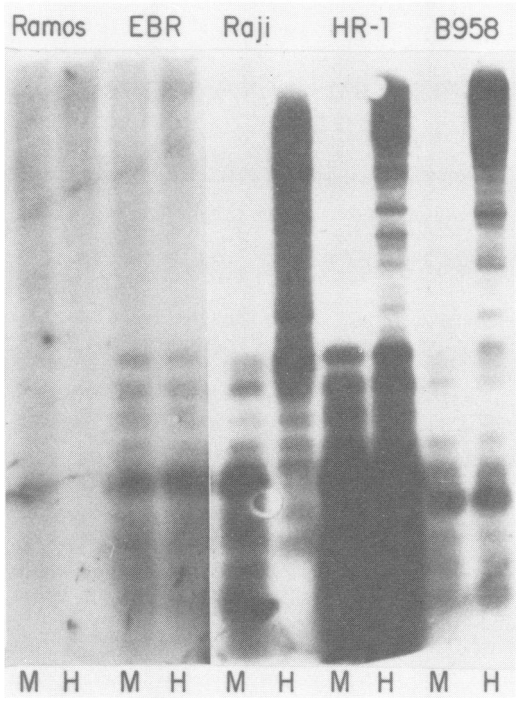

FIG. 1. EBV DNA sequences in *Msp*I (M) and *Hpa*II (H) digests of whole cell DNA from EBV host cell cultures. Whole cell DNA was isolated from Ramos, EBR, Raji, HR-1, and B958 cells and purified by phenol extraction or CsCl density gradient centrifugation (2, 21). Restriction enzyme digestions were performed with *Msp*I and *Hpa*II (New England Biolabs) at 37°C in the presence of buffer containing 20 mM Tris (pH 7.8), 10 mM $MgCl_2$, and 10 mM mercaptoethanol. Digestions were for a minimum of 3 h in the presence of an eightfold excess of enzyme. To determine that *Hpa*II digestions were complete we performed similar experiments, digesting these DNAs with twice the amount of enzyme (16-fold excess). They were found to have electrophoretic patterns identical to those shown here. The digested whole cell DNAs were precipitated with ethanol, and 10 μg of each was electrophoresed in 1.2% horizontal agarose gels with a buffer of 100 mM Tris-borate (pH 8.3)–25 mM EDTA (15). Electrophoresed DNA digests were transferred to nitrocellulose paper (23) and hybridized (19) with a probe of EBV HR-1 virion DNA (Showa University Research Institute/National Cancer Institute) which had been nick-translated with [α-^{32}P]dCTP (18).

restriction enzyme digestion at unmethylated CCGG sites should be present in both *Msp*I and *Hpa*II digests. By this criterion part of the EBV DNA in digests of DNA from producer cell lines HR-1 and B958 was unmethylated at the internal cytosine of all CCGG sites cut by *Msp*I. In addition, both digests contained EBV DNA which was methylated at CCGG, as indicated by the presence of higher-molecular-weight viral DNA fragments in the *Hpa*II digests. DNA from the nonproducer cell line Raji, on the other hand, contained EBV DNA which was cut well by *Msp*I but very little by *Hpa*II, indicating that the EBV DNA present in Raji cells was highly methylated at CCGG sites. DNA from the EBV-negative Ramos cell line did not hybridize with the EBV DNA probe, showing that the probe was not contaminated with cellular DNA at a detectable level.

Of greatest interest was the observation that the viral DNA of the EBR cell line was unmethylated at CCGG sites despite the nonproducer status of these cells.

The methylation of sites in EBV other than CCGG was studied by repeating the blotting hybridization experiments described above but digesting the whole cell DNAs with other restriction enzymes which recognized unmethylated C-G sites. In contrast to the EBV DNA in the other cell lines tested, the EBV DNA in the nonproducer line EBR was completely unmethylated at *Hha*I (GCGC) and *Hae*II (PuGCGCPy) sites as well as at the *Hpa*II site. The only C-G-containing site that showed a detectable level of methylation in EBV DNA in EBR cells was that of *Sst*II (CCGCGG). These data, including the results shown in Fig. 1, are summarized in Table 1.

Some aspects of our data are different from the data previously reported for herpesvirus saimiri, in which three different producer cell lines were found to have unmethylated viral DNA (5). The data reported here indicate that EBV from two producer cell lines was present as both methylated and totally nonmethylated genomes. The simplest explanation for the presence of both methylated and nonmethylated

TABLE 1. Summary of comparative degree of digestion of various EBV DNAs by restriction endonucleases[a]

Enzyme	Recognition site	Digestion[b] of EBV-containing cell line:			
		EBR	Raji	HR-1	B958
*Msp*I	CCGG, CM^{5}CGG	+	+	+	+
*Hpa*II	CCGG	+	(−)	(+)	(−)
*Hha*I	GCGC	+	(−)	(+)	(+)
*Hae*II	PuGCGCPy	+	(−)	(+)	(−)
*Sst*II	CCGCGG	(−)	−	(+)	(−)

[a] Experiments utilizing *Hha*I, *Hae*II, and *Sst*II were done as described in the legend to Fig. 1 and in the text.

[b] +, Full or almost full digestion; (+), much DNA digested but significant uncut DNA population remained; (−), most DNA undigested but cut DNA apparent; −, little or no digestion.

viral DNA lies in the nature of EBV-transformed producer cell lines. In these lines, only a small percentage of the cell population actively produces virus at any one time although all of the cells in the population carry EBV DNA. Therefore the nonmethylated viral DNA would most likely be from the producer portion of the cell population and the methylated viral DNA would be from the nonproducer cell population.

Since the EBR line is reported to be a nonproducer line in that there are only a few viral genome copies per cell and no viral capsid antigen has been detected (8; Clough and Dougherty, unpublished data), we would predict that the viral DNA from this cell line would be highly methylated, as is the viral DNA in Raji cells. However, our data show that EBV DNA in EBR is totally nonmethylated at three C-G-containing sites which were at least partially methylated in the viral DNA of the other cell lines tested. EBR is the only cell line examined in this report that was not originally transformed by EBV. This difference in origin may be useful information in planning experiments to determine the mechanisms of herpesvirus genome repression in this nonproducer cell line.

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